REMARKS

Claims 3-6, 11, 13-16 and are pending in the present application and are under examination. Applicant reserves the right to pursue any canceled subject matter in a future application.

The Office Action asserts that Applicants have not complied with the requirement of 35 U.S.C. 120 for allegedly failing to include a reference to parent application serial no. 08/400,796.

Applicants submit that at the filing of the instant application on November 5, 1997, the TRANSMITTAL OF FILING UNDER 37 CFR 1.60(b) indicated on the top of page 2 that this is a divisional application under 37 CFR 1.60 of the then co-pending parent application serial number 08/400,796. Also on page 5, section 9 (titled "Related Back – 35 USC 120"), Applicants amended "the specification by inserting before the first line the sentence: 'This is a divisional of copending applications Serial number 08/400,796 filed on March 7, 1995.'" Therefore, the requirement of 35 USC 120 for claiming the benefit of an earlier application is met. Nevertheless, Applicants have amended the sentence to update the status of the parent application. Reconsideration and withdrawal of the objection are respectfully requested.

Applicants respectfully request reconsideration in view of the following remarks. Issues raised by the Examiner will be addressed below in the order they appear in the Office Action.

Claim rejections under 35 U.S.C. 112, first paragraph – Written Description

Claims 3-6, 11, and 13-16 remain rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the invention(s), at the time the application was filed, had possession of the claimed invention.

The Office Action alleges that the term "consisting essentially of" is construed to be equivalent to "comprising," "absent a clear indication in the specification or claims of what the basic and novel characteristics actually are." To support this position, the Office Action insists that there are positions of the sequence motif with unrestricted amino acid sequences, which may be deleterious to binding. Specifically, the Office Action asserts that the presence of putative binding motif residues does not necessarily correlate with actual binding to MHC proteins; that amino acid residues outside the "core" may be deleterious to binding; and that there is no written

description of amino acids that constitute the TCR binding site. Finally, the Office Action concludes that skilled artisans cannot envision the detailed structures of the encompassed peptide, "and therefore conception is not achieved until reduction to practice has occurred."

While not acquiescing to the reasoning of the Office Action, to expedite prosecution, Applicants have amended claims 3, 6, 13, and 16, to clarify the subject matter claimed. Applicants submit that all claims currently pending meet the written description requirement of 35 U.S.C. 112, first paragraph, since the amended claims clearly recite a defined structural element associated with a function. Applicants submit that the Office Action's rejection regarding the term "consisting essentially of" is rendered moot in view of these amendments.

Regarding the Office Action's concern that there are positions in the sequence motif that may have undefined sequences, such that certain sequences fitting a formula (sequence motif in this case) may not necessarily have the requisite functional characteristics, Applicants submit that the pending claims couple functional limitations with structural elements, and thus comply with the "Revised Interim Written Description Guidelines Training Materials" of the Office.

Applicants wish to draw the Examiner's attention to Example 9 of the "Revised Interim Written Description Guidelines Training Materials" obtained from the USPTO website (a copy of Example 9 is submitted herewith as **Exibit A**). In Example 9 of the Guideline, the hypothetical claim is directed to a genus of nucleic acids, all of which must hybridize under high stringency conditions with SEQ ID NO: 1, and must encode a protein with a specific activity (binding to a dopamine receptor and stimulating adenylate cyclase activity). Since the hypothetical SEQ ID NO: 1 is novel and fully disclosed, and falls within the scope of the hypothetical claim, the single species meets the written description requirement. As to the genus claim, the Guideline further elaborates that "a person of skill in the art would not expect substantial variation among species encompassed within the scope of the claims because the highly stringent hybridization conditions set forth in the claim yield structurally similar DNAs. Thus a representative number of species is disclosed...and the level of skill and knowledge in the art are adequate to determine that applicant was in possession of the claimed invention." Thus the conclusion of the Guideline is that the claimed invention is adequately described.

In the above example, not all polynucleotides that can hybridize with the hypothetical SEQ ID NO: 1 are within the scope of the genus claim, precisely because certain hybridizing

polynucleotides may not encode an polypeptide at all, or encode polypeptides without the requisite functional characteristics.

In the instant case, not all polypeptide sequences that fit the sequence motifs are within the scope of the claims, depending on whether or not such polypeptides meet the functional criteria set forth in the claims. Some polypeptides with putative binding motif residues may possibly be non-binders, because of the presence of amino acid residues, such as those outside the "core," that might even be deleterious to MHC binding. But that does not render the claims defective in terms of meeting the written description requirement, for the same reasons the hypothetical claim in Example 9 is not defective.

Regarding the written description for certain TCR binding residues of the sequence motifs, page 15, 1st paragraph describes P-1, P2, P3, P5, P8 and P11 as TCR contact residues. Pages 18-19 further describe the situations where restrictions at the TCR contact residues would or would not be necessary. Applicants submit that specific sequences at positions such as P2, P3 and P5 (putative TCR contacting residues) may or may not need to be defined in certain cases since the functional limitation requiring the claimed polypeptide to be able to bind MHC molecule and activate autoreactive T cells from patient inherently / implicitly restricts the possible choices of such TCR contacting residues. This is similar to Example 9 above, wherein specific sequences within the scope of the genus claim, other than the hypothetical SEQ ID NO: 1, need not be spelled out since the functional limitation of dopamine binding and Adenylate Cyclase activation would serve to restrict sequence variation of polypeptides within the claim scope. Secondly, in MS motifs #1-3, certain TCR contact residues <u>are</u> restricted according to the teaching of the specification. Thus contrary to the Office Action's assertion, there is adequate written description in the specification of the TCR contact residues <u>where appropriate</u>.

In fact, for reasons described in pages 18-19, certain TCR contacting sites probably should not be limited in the sequence motif. For example, in the PV example disclosed in the specification, it was previously known only that the human desmoglein 3 protein (DG) is the autoantigen, but there was no specific knowledge of which of the many possible epitopes within the DG protein might be causing the autoimmune response. Indeed, the specification has identified several such DG epitopes, each of which, when presented by the PV-associated HLA-DR4, is expected to bind to a <u>different</u> TCR, thus involving different T cell contact residues. This

is also confirmed by the fact that two of the seven identified epitopes specifically activate proliferation of autoimmune patient T cells (page 38, last paragraph). In a subsequent article published by the inventors, two additional identified peptides are shown to react with autoreactive T cells from other PV patients (see page 11937, Table 3 of Wucherpfennig et al., Proc. Natl. Acad. Sci. U.S.A. 92: 11935-39, 1995, **Exhibit B**). This also demonstrates that different patients may have different (subsets of) autoreactive T-cells (e.g., patient 1 may have T cells recognizing PVA.3 and PVA.4; patient 2 may only have T cells recognizing PVA.7, etc.), thus a negative result in a limited number of tested patients (such as 2 in the instant specification, and 4 in the Wucherpfennig article) may not necessarily mean that an identified autoreactive epitope cannot activate any autoreactive T-cell from some (as yet untested) patients. Thus, the scope of the claims should not be limited to the Examples provided in the instant specification.

In contrast, in the MS example, the 15-mer MBP epitope spanning residues 85-99 (unlike the *whole* DG protein in PV) is the known epitope. Thus specific TCR binding such a defined epitope must have relatively conserved sequences, depending on the specific presenting MHC subtypes (see page 42 for MS motif #2 design) and/or known subgroups of TCRs specific for the epitope (see page 42-43 for MS motif #3 design). In those cases, the instant specification not only provides adequate written description of the restricted TCR contact sites themselves in the sequence motifs, but also a scientific reasoning supported by experimental data behind such choices. Thus the written description requirement is met.

The Office Action is also of the opinion that WO 94/06828, referred to on page 30 at lines 15-18 as a support for using high dose antigens to tolerize a host animal, does not actually provide such support. Regardless, Applicants submit that the phenomenon of using high dose antigen to induce anergy in the immune system is a well-known phenomenon and frequently used practice in the field of immunology and vaccination at the time of the instant invention, thus no specific description of such a method is needed. "A patent need not teach, and preferably omits, what is well known in the art." (MPEP 2164.01).

The Office Action further asserts that "the specification does not disclose the greater than 70 known HLA-DR allotypes, nor their pocket structures or motifs for peptides that bind to them, and association with susceptibility to autoimmune diseases." Without addressing the

merits of these allegations, Applicants submit that amendments to the pending claims render these arguments moot.

The Office Action also cited several recent CAFC cases (The Regents of the University of California v. Eli Lilly and Co.; Amagen, Inc. v. Chugai Pharmaceutical Co., Ltd.; and Fiers v. Revel) to support its positions. Applicants note that the "Revised Interim Written Description Guidelines Training Materials" described above was drafted in view of, rather than in conflict with, these established case law decisions. The position of the USPTO as represented in the Guideline is consistent with such decisions, and has not been overruled by any court. The pending claims are also consistent with the established practice of the USPTO to couple structural elements to functional characterizations.

Therefore, Applicants submit that all pending claims as presented meet the written description requirement of 35 U.S.C. 112, first paragraph. Reconsideration and withdrawal of the rejection are respectfully requested.

Claim rejections under 35 U.S.C. 112, first paragraph - Enablement

Claims 3-6, 11, and 13-16 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. The Office Action alleges that the specification is only enabling for human polypeptide consisting of one of SEQ ID NOs: 1-7, and thus the pending claims are not commensurate in scope.

The Office Action repeated verbatim some reasoning used in the written description rejection – certain positions of the sequence motifs might be undefined, and thus there could be non-binders with putative sequence motifs. Applicants point out that written description and enablement are distinct requirements, and reasoning for written description defects may not necessarily relate to enablement defects. Particularly in view of the amendments, a skilled artisan could easily make the claimed pharmaceutical preparation according to the disclosed sequence motifs and functional assays, as exemplified by the two detailed examples.

The Office Action cites Chicz, which allegedly disclose that *naturally processed* peptides could be between 10-34 amino acids in length. Also, it is alleged that skilled artisans knew, at the

time of the instant invention, that class II MHC / HLA molecules are capable of binding larger exogenous peptides. Therefore, the Office Action concludes that in long polypeptides, amino acid residues outside of the core residues would render the polypeptide susceptible to other frames of binding to the HLA molecule than the intended frame consisting of the motif amino acid residues. However, the Office Action does not explain why this allegation, even if true, renders the claimed invention not enabled.

Assuming (without admitting) that it is true that additional residues outside the core binding regions may "stick out" from the binding groove of the class II MHC molecule, and that such larger polypeptides might render the polypeptide susceptible to other frames of binding to the HLA molecule than the intended frame of binding. Under this circumstance, the larger polypeptide likely binds to an HLA molecule other than the one recognizing the intended frame of binding (otherwise, it is the same situation as different DG protein epitopes binding to the same disease-associated HLA-DR4 molecule as disclosed in the specification). It is the latter HLA that is known to be associated with the autoimmune disease in question. In addition, if such "accidental" binding to such other unintended HLA molecule does exist, such other unintended HLA molecule may well be associated with the same autoimmune disease (if it is not, then the binding is immaterial to the claimed subject matter, and is outside the scope of the claimed invention, which requires binding to *autoimmune disease-associated HLA molecules*). In this case, the long polypeptide with two frames of binding ("intended" and "unintended" with respect to one disease-associated HLA) might just be one claimed polypeptide that happens to be able to bind two HLA molecules, both associated with the same autoimmune disease. A skilled artisan, after seeing such overlap in sequences, is enabled to make either a fragment of such a long polypeptide that binds to one of the two HLAs, or the long polypeptide that binds to both HLAs.

The Office Action also alleges that "there is no guidance in the specification so to what alterations resulting in a functional polypeptide, i.e., one that binds to HLA-DR (...) and to a TCR and causes tolerization." Thus, the Office Action asserts that undue experimentation is required for a skilled artisan to determine such substitutions/additions to make the claimed polypeptide.

Applicants submit that no such knowledge regarding specific residue alterations is needed to make the claimed invention. A skilled artisan *can*, but does not *have to* make a

molecule functionally equivalent to a known one by using a blue print-like "map" indicating which residues of the original molecule can be changed to what other residues. Instead, a much more efficient and cost-effective way of making such functionally equivalent molecules is by complete or partial random mutagenesis/synthesis, followed by a functional screen. For example, by fixing the P1, P4 and P6 positions to the few restricted residues according to the relevant sequence motif, and allowing all 20 amino acids at other positions of a, say, 15-mer polypeptide, a mixture of such (synthesized) polypeptides can be passed through an immobilized column of a disease-associated HLA molecules, which only binds those polypeptides within the scope of the claimed invention.

This is much like the situation of those equivalent polynucleotides in the hypothetical claim in Example 9 of the Guideline – given SEQ ID NO: 1, a skilled artisan *could*, but does not *have to* make those other polynucleotides that hybridize under high stringency conditions to SEQ ID NO: 1, based on a "blue print" indicating which nucleotides can be substituted by which other nucleotides to make a functionally equivalent molecule. To require a patentee to describe such thousands, if not millions of sequences in detail for the purpose of making them is not only unreasonable but also unnecessary.

The Office Action next holds the position that the recited "HLA-DR protein" encompasses hundreds of HLA-DR proteins that may be associated with many disclosed and undisclosed autoimmune diseases. Applicants submit that the amended claims recite specific autoimmune diseases (PV or MS) and specific HLA molecules associating therewith. The specification teaches that only one or few specific alleles of the HLA molecules are presently known to be associated with such autoimmune diseases.

The Office Action also raises the issue that the claims may encompass polypeptides with residues that may or may not provide adequate T cell receptor contact. As explained above, it may or may not be proper to specify TCR contact sites depending on specific circumstances (such as whether it is known that certain antigens or epitopes are known HLA binders). Also as explained above, there is no need to know the sequence of these TCR contact residues, under certain proper circumstances, to make the claimed polypeptide using, for example, the screening method described above.

The Office Action also holds the position that polypeptides longer than 15 amino acids can be bound by class II MHC molecules. This may be true to a certain extent in view of Chicz, and Applicants have addressed above why this does not in any way render the claimed invention not enabled.

The Office Action also alleges that the disclosed several positions (e.g., 3 out of 5) for binding to particular MHC class II molecules may not necessarily be predictive of binding and "not of which residues at non-MHC class II contact positions are those that are responsible for T cell receptor contact and tolerization." Applicants have addressed above that the structural element (sequence motif) is coupled to the functional assay to define the claimed invention, thus any polypeptide within the scope of the invention must be able to bind and activate TCR.

The Examiner also takes the position, without substantiating by citing relevant scientific article/evidence, that a skilled artisan was aware that it is unpredictable in whether tolerization can be produced in vivo despite efficient recognition in vitro. Pursuant to MPEP 707.05 and 37 CFR 1.104(d)(2), Applicants respectfully request the Examiner to provide support for her position by providing such references or an affidavit.

Finally, the Examiner holds the position, without substantiating by citing relevant scientific article/evidence, that a skilled artisan would have been aware that tolerization with a preparation comprising a protein or peptide can be dependent upon route of administration and dosage and timing of dosing. Pursuant to MPEP 707.05 and 37 CFR 1.104(d)(2), Applicants respectfully request the Examiner to provide support for her position by providing such references or an affidavit. In addition, even assuming the truth of this allegation, Applicants submit that this position *per se* does not render the claimed invention not enabled, since variations in route of administration, dosage and timing do not necessarily implicating undue experimentation. In fact, a tending health worker such as a physician would need to decide based on patient-specific factors in most situations, including patient's weight, age, gender, health condition, past pre-existing condition, allergic reaction, etc., and such evaluations are routine, not undue.

Therefore, Applicants submit that all pending claims are enabled and to the full extent. Reconsideration and withdrawal of the rejection are respectfully requested.

Claim rejections under 35 U.S.C. 112, second paragraph

Claim 13-16 remain rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

To avoid potential confusion, Applicants have amended claim 13 to clarify the subject matter being claimed. Amended claim 13 unequivocally recites an immunogenic antigen from a human pathogen, which antigen is also effective to immunize against that pathogen. This effectively clarifies the confusion in paragraph 7b of the office Action. Amended claim 13 also indicates that the antigen excludes / does not comprise any polypeptide that contains the sequence motif for the disease-associated HLA-DR protein, see page 8, 1st paragraph ("[t]he preparations of the present invention specifically do not include such polypeptides but, rather, include other antigens from the pathogen.")

The issue of "native form" is also rendered moot due to this amendment.

Thus, reconsideration and withdrawal of the rejection under 35 U.S.C. 112, second paragraph, are respectfully requested.

Double Patenting Rejections

The Office Action states that claims 3-6, and 13-16 stand rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claim 3 of the parent U.S. Application serial number 08/400,796, now U.S. Pat. No. 5,874,531.

Applicants note that, pursuant to 37 CFR 1.130(b), a terminal disclaimer in compliance with 37 CFR 1.321(c) may be used to overcome the double patenting rejection. Applicants will submit a terminal disclaimer, if necessary, upon indication of allowable subject matter.

Claim rejections under 35 USC §102

The Office Action states that claims 3-5, and 13-15 stand rejected under 35 USC 102(b) as being anticipated by Amagai et al. for the reasons of record.

While not acquiescing in the reasoning of the Office Action, to expedite prosecution, Applicants have amended claims 3 and 13 (and their dependent claims) to clarify the subject

matter claimed. Applicants submit that the claims as amended are not anticipated by Amagai, since the amended claims do not read on the full-length human desmoglein 3 protein, even if, for the sake of argument, the reasoning of the Office Action is applied.

Regarding the assertion of the Office Action about claim 13 reciting "a polypeptide having an amino acid sequence motif for an HLA-DR protein...," Applicants submit that this issue appears to arise from a confusion and misinterpretation of the claim language, but is now rendered moot in view of the amendment to claim 13.

In addition, Applicants do not understand the relevance of the administration route, timing and dosing in the context of prior art anticipation. Clarification is respectfully requested.

Therefore, reconsideration and withdrawal of the rejection under 35 U.S.C. 102(b) are respectfully requested.

CONCLUSION

In view of the foregoing amendments and remarks, Applicants submit that the pending claims are in condition for allowance. Early and favorable reconsideration is respectfully solicited. The Examiner may address any questions raised by this submission to the undersigned at 617-951-7000. Should an extension of time be required, Applicants hereby petition for same and request that the extension fee and any other fee required for timely consideration of this submission be charged to **Deposit Account No. 18-1945.**

Respectfully Submitted,

Date: June 17, 2004

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REVISED INTERIM WRITTEN DESCRIPTION GUIDELINES TRAINING MATERIALS

Contents

SYNOPSIS OF APPLICATION OF WRITTEN DESCRIPTION GUIDELINES

It is assumed at this point in the analysis that the specification has been reviewed and an appropriate search of the claimed subject matter has been conducted. It is also assumed that the examiner has identified which features of the claimed invention are conventional taking into account the body of existing prior art. There is a strong presumption that an adequate written description of the claimed invention is present in the specification as filed. If the examiner determines that the application does not comply with the written description requirement, the examiner has the initial burden, after a thorough reading and evaluation of the content of the application, of presenting evidence or reasons why a person skilled in the art would not recognize that the written description of the invention provides support for the claims. It should also be noted that the test for an adequate written description is separate and distinct from the test under the enablement criteria of 35 U.S.C. § 112 first paragraph. The absence of definitions or details for well-established terms or procedures should not be the basis of a rejection under 35 U.S.C. 112, para. 1, for lack of adequate written description. Limitations may not, however, be imported into the claims from the specification.

The following examples only describe how to determine whether the written description requirement of 35 U.S.C. 112, para. 1 is satisfied. Regardless of

the outcome of that determination, Office personnel must complete the patentability determination under all the relevant statutory provisions of Title 35 of the U.S. Code. Once Office personnel have concluded analysis of the claimed invention under all the statutory provisions, including 35 U.S.C. 101, 112, 102, and 103, they should review all the proposed rejections and their bases to confirm their correctness. Only then should any rejection be imposed in an Office action. The Office action should clearly communicate the findings, conclusions, and reasons which support them. When possible, the Office action should offer helpful suggestions on how to overcome rejections.

e.g. expression vectors, the necessary common attribute is the ORF (SEQ ID NO: 2).

Weighing all factors including (1) that the full length ORF (SEQ ID NO: 2) is disclosed and (2) that any substantial variability within the genus arises due to addition of elements that are not part of the inventor's particular contribution, taken in view of the level of knowledge and skill in the art, one skilled in the art would recognize from the disclosure that the applicant was in possession of the genus of DNAs that comprise SEQ ID NO: 2.

Conclusion: The written description requirement is satisfied.

Example 9: Hybridization

Specification: The specification discloses a single cDNA (SEQ ID NO:1) which encodes a protein that binds to a dopamine receptor and stimulates adenylate cyclase activity. The specification includes an example wherein the complement of SEQ ID NO: 1 was used under highly stringent hybridization conditions (6XSSC and 65 degrees Celsius) for the isolation of nucleic acids that encode proteins that bind to dopamine receptor and stimulate adenylate cyclase activity. The hybridizing nucleic acids were not sequenced. They were expressed and several were shown to encode proteins that bind to a dopamine receptor and stimulate adenylate cyclase activity. These sequences may or may not be the same as SEQ ID NO: 1.

Claim:

An isolated nucleic acid that specifically hybridizes under highly stringent conditions to the complement of the sequence set forth in SEQ ID NO: 1,

wherein said nucleic acid encodes a protein that binds to a dopamine receptor and stimulates adenylate cyclase activity.

Analysis:

A review of the full content of the specification indicates that the essential feature of the claimed invention is the isolated nucleic acid that hybridizes to SEQ ID NO: 1 under highly stringent conditions and encodes a protein with a specific function. The art indicates that hybridization techniques using a known DNA as a probe under highly stringent conditions were conventional in the art at the time of filing.

The claim is drawn to a genus of nucleic acids all of which must hybridize with SEQ ID NO: 1 and must encode a protein with a specific activity.

The search of the prior art indicates that SEQ ID NO: 1 is novel and unobvious.

There is a single species disclosed (a molecule consisting of SEQ ID NO: 1) that is within the scope of the claimed genus.

There is actual reduction to practice of the disclosed species.

Now turning to the genus analysis, a person of skill in the art would not expect substantial variation among species encompassed within the scope of the claims because the highly stringent hybridization conditions set forth in the claim yield structurally similar DNAs. Thus, a representative number of species is disclosed, since highly stringent hybridization conditions in combination with the coding function of DNA and the level of

skill and knowledge in the art are adequate to determine that applicant was in possession of the claimed invention.

Conclusion: The claimed invention is adequately described.

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Immunology

Structural basis for major histocompatibility complex (MHC)-linked susceptibility to autoimmunity: Charged residues of a single MHC binding pocket confer selective presentation of self-peptides in pemphigus vulgaris

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Contributed by Jack L. Strominger, September 14, 1995

ABSTRACT **Human T-cell-mediated autoimmune diseases are genetically linked to particular alleles of MHC class II genes. Susceptibility to pemphigus vulgaris (PV), an autoimmune disease of the skin, is linked to a rare subtype of HLA-DR4 (DRB1*0402, 1 of 22 known DR4 subtypes). The PV-linked DR4 subtype differs from a rheumatoid arthritis-associated DR4 subtype (DRB1*0404) only at three residues (DRβ 67, 70, and 71). The disease is caused by autoantibodies against desmoglein 3 (DG), and T cells are thought to trigger the autoantibody production against this keratinocyte adhesion molecule. Based on the DRB1*0402 binding motif, seven candidate peptides of the DG autoantigen were identified. T cells from four PV patients with active disease responded to one of these DG peptides (residues 190–204); two patients also responded to DG-(206–220). T-cell clones specific for DG-(190–204) secreted high levels of interleukins 4 and 10, indicating that they may be important in triggering the production of DG-specific autoantibodies. The DG-(190–204) peptide was presented by the disease-linked DRB1*0402 molecule but not by other DR4 subtypes. Site-directed mutagenesis of DRB1*0402 demonstrated that selective presentation of DG-(190–204), which carries a positive charge at the P4 position, was due to the negatively charged residues of the P4 pocket (DRβ 70 and 71). DRβ 71 has a negative charge in DRB1*0402 but a positive charge in other DR4 subtypes, including the DR4 subtypes linked to rheumatoid arthritis. The charge of the P4 pocket in the DR4 peptide binding site therefore appears to be a critical determinant of MHC-linked susceptibility to PV and rheumatoid arthritis.**

The MHC is an important susceptibility locus for human autoimmune diseases, such as insulin-dependent diabetes, rheumatoid arthritis (RA), and pemphigus vulgaris (PV) (reviewed in refs. 1–4). A genome-wide search for type I diabetes susceptibility genes with microsatellite markers confirmed that the MHC is the most important susceptibility locus and that a number of other genes contribute to the disease process (5). Disease-linked polymorphisms map to the peptide binding site of MHC molecules (6, 7), indicating that peptide presentation to T cells may be important in the initiation or progression of these diseases. The HLA-DR4-linked autoimmune diseases, RA and PV, may offer an opportunity to define the structural basis of MHC-linked disease susceptibility. Susceptibility to autoimmunity in RA and PV is linked to MHC class II molecules that differ only in a small, defined region of the peptide binding site (3, 4, 8–10).

PV is a life-threatening blistering disease of the skin and mucous membranes (11). Autoantibodies specific for a keratinocyte cell adhesion molecule, desmoglein 3 (DG), cause a loss of cell adhesion and blister formation. DG is a member of the cadherin family of Ca^{2+}-dependent cell adhesion molecules (12). The autoantibodies are pathogenic as neonates of mothers with PV have a transient blistering skin disease due to maternal IgG that crosses the placenta (13). A loss of cell adhesion and blister formation are observed when IgG or affinity-purified DG-specific antibodies from PV patients are transferred to neonatal mice by i.p. injection (14–16).

Susceptibility to PV is linked to the DRB1*0402 haplotype, 1 of 22 known DR4 haplotypes. Among Ashkenazi Jews, >90% of PV patients carry the DRB1*0402 haplotype, which is rare in the general population. The DQ8 genes (DQA1*0301, DQB1*0302) of this haplotype are also found in haplotypes not associated with susceptibility to PV (i.e., DRB1*0401), indicating that the primary association is with the DRB1*0402 gene (9, 17, 18).

In other ethnic groups (non-Ashkenazi Jews, Caucasians, Japanese) susceptibility is linked to a rare DQ1 allele (DQB1*0503) (17, 19, 20). The DQB1*0503 allele is structurally very interesting since it differs from a common DQB1 allele (DQB1*0501) only by a valine to aspartic acid substitution of DQβ 57. The DQβ 57 position is also important in the diabetes-linked DQ alleles (DQB1*0302, DQB1*0201). In contrast to the PV-linked DQ allele, where Asp-57 *confers* susceptibility, susceptibility to diabetes is linked to alleles that do *not* carry a negative charge at DQβ 57 (2, 21, 22). Polymorphisms of particular MHC class II pockets (P4 pocket in DR4 molecules, P9 pocket in DQ molecules) therefore appear to be important in determining susceptibility to several different human autoimmune diseases.

MATERIALS AND METHODS

Methods for generating human T-cell clones and for examining their MHC/peptide specificity were as described (23).

RESULTS

Structural Motif for Peptides Presented by the PV-Associated DR4 Molecule. The PV-associated DR4 subtype (DRB1*0402) carries a negative charge at DRβ 70 and 71; in the DR1 crystal structure these residues are located in the P4 pocket of the peptide binding site. Most DR4 subtypes (except DRB1*0402 and 0414) have a positive charge (lysine or arginine) at DRβ 71. Residues that are polymorphic between the DR4 subtypes are listed for DRB1*0401 to 0412 in Table 1; the other known DR4 subtypes (DRB1*0413 to 0422) are

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: MHC, major histocompatibility complex; PV, pemphigus vulgaris; IL, interleukin; RA, rheumatoid arthritis; DG, desmoglein 3; r, recombinant; EBV, Epstein–Barr virus.

Table 1. Polymorphic residues of HLA-DR4 subtypes

	DRβ	0401	0402	0403	0404	0405	0406	0407	0408	0409	0410	0411	0412
	37	Tyr	Tyr	Tyr	Tyr	Tyr	Ser	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr
P9 pocket	57	Asp	Asp	Asp	Asp	Ser	Asp	Asp	Asp	Ser	Ser	Ser	Ser
	67	Leu	Ile	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Ile
	70	Gln	Asp	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Asp
P4 pocket	71	Lys	Glu	Arg	Arg	Arg	Arg	Arg	Arg	Lys	Arg	Arg	Arg
	74	Ala	Ala	Glu	Ala	Ala	Glu	Glu	Ala	Ala	Ala	Glu	Leu
P1 pocket	86	Gly	Val	Val	Val	Gly	Val	Gly	Gly	Gly	Val	Val	Val

The PV-associated DR4 molecule (DRB1*0402) has a negative charge at DRβ 70 and 71; other DR4 subtypes have a positive charge at DRβ 71 (lysine or arginine). The DR4 molecules associated with RA (DRB1*0401 and 0404) therefore have a positively charged P4 pocket, while the PV-associated DR4 molecule (DRB1*0402) has a negatively charged P4 pocket.

very rare (24). DRB1*0402 and 0414 differ only by the Val/Gly dimorphism that controls the size of the P1 pocket.

The comparison of DRB1*0402 (PV linked) and 0404 (RA linked) is particularly informative as these alleles differ only at three positions (DRβ 67, 70, and 71). DRB1*0404 confers an increased risk for RA, indicating that the DRβ 67–71 segment is critical for determining susceptibility to these two different autoimmune diseases (8, 18).

The crystal structure of HLA-DR1 demonstrated that DRβ 70 and 71 contribute to the shape and charge of a pocket that accommodates the P4 side chain of the bound peptide (the P1 side chain is the first DR anchor residue) (7) (Fig. 1). This suggested that DRβ 70 and 71 may confer an increased risk to RA and PV by allowing the binding of self-peptides with a particular shape/charge at P4. Peptide binding studies demonstrated that peptides with a charge complementary to that of DRβ 71 were selective for either the RA- or the PV-associated DR4 molecules (10). Based on these considerations and on the previously established preferences for the P1 and P6 pockets (25), a set of criteria was developed for the identification of candidate peptides from the known target antigen, DG (4). The DRB1*0402 binding criteria considered the preference for hydrophobic residues (V, L, I, M, F) at P1, small side chains at P6 (S, T, N, V), and positively charged residues at P4 (K, R). Seven peptides of DG matched this structural motif (Table 2). Six of these peptides were located in the extracellular domain that is recognized by DG-specific autoantibodies (12).

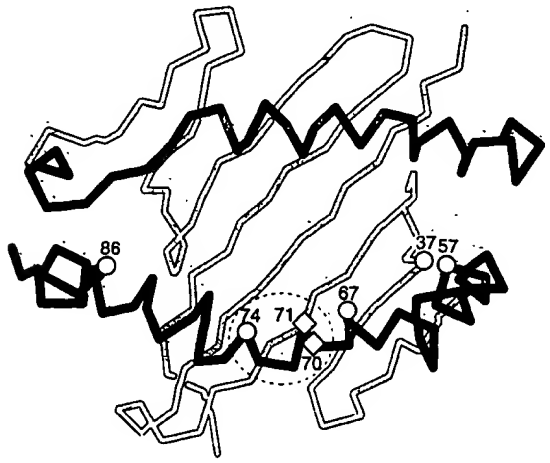

FIG. 1. Position of polymorphic DRβ chain residues in the HLA-DR peptide binding site. Residues DRβ 70 and 71 have a negative charge in the pemphigus-associated DRB1*0402 molecule and are located in the P4 pocket (circled) (7). DRB1*0402 and the closely related 0414 are the only DR4 alleles that have negatively charged residues at DRβ 70 and 71 of the P4 pocket. All other DR4 subtypes have a positive charge at DRβ 71. The negative charge of DRβ 70 and 71 is responsible for the selective presentation of self-peptides with a positive charge at P4.

Recognition of DG Peptides by T Cells from PV Patients. In PV, the clinical course is marked by relapses and remissions; disease activity correlates with the production of DG-specific autoantibodies (12, 15) against an immunodominant epitope of the extracellular domain, residues 200–229 (16). T-cell responses to the seven DG peptides were evaluated in four patients with active disease. Patients had clinical features of PV; the diagnosis was confirmed by histology and immunohistology of the skin. T-cell lines were raised against the DG peptides from blood mononuclear cells using peptides at a concentration of 5 μM. T-cell lines were expanded by addition of recombinant interleukin 2 (rIL-2) and tested for their specificity after 12–14 days of culture; T-cell lines with a stimulation index of >3 [T-cell proliferation in the presence of peptide (cpm)/T-cell proliferation in the absence of peptide (cpm)] were considered positive. From each patient, DG-(190–204)-specific T-cell lines were obtained that showed a strong proliferative response in the initial assay (stimulation index >10). Two patients also showed a response to the DG-(206–220) peptide and one patient responded to DG-(251–265) or DG-(762–786). No T-cell responses were seen to peptides DG-(78–93), DG-(97–111), and DG-(512–526) (Table 3). These data demonstrate that at least one DG peptide (residues 190–204) is a target for autoreactive T cells in PV patients with active disease. The DG-(190–204) and DG-(206–220) T-cell epitopes are adjacent to each other in the extracellular domain of DG and overlap with an immunodominant antibody epitope of the extracellular domain, DG-(200–229) (16).

It is not yet known whether the DG-(190–204) peptide is the immunodominant T-cell epitope of DG. This question may be

Table 2. The binding motif of the PV-associated DR4 molecule (encoded by DRA, DRB1*0402 genes) was used to define peptides of the DG autoantigen

Motif	1	4	6
	V	K	S
	L	R	T
	I		N
	M		V
	F		
DG-(78–93)	ATQK**I**TY**R**I**S**GVGID		
DG-(97–111)	FGIF**V**VD**K**N**T**GDINI		
DG-(190–204)	LNSK**I**AF**K**I**V**SQEPA		
DG-(206–220)	TPMF**L**LS**R**N**T**GEVRT		
DG-(251–265)	CECN**I**KV**K**D**V**NDNFP		
DG-(512–526)	SART**L**NN**R**Y**T**GPYTF		
DG-(762–786)	QSGT**M**RT**R**H**S**TGGTN		

In this motif, the MHC anchor positions P1, P4, and P6 were considered. Peptide residue P4 carries a positive charge (K or R) due to the negative charge of the P4 pocket in the PV-associated DR4 molecule.

Table 3. T-cell responses to DG peptides in PV patients

	Patient Go		Patient Ro		Patient DC		Patient St	
Peptide	No peptide	Plus peptide	No peptide	Plus peptide	No peptide	Plus peptide	No peptide	Plus peptide
DG-(78–93)	—	—	—	—	—	—	—	—
DG-(97–111)	—	—	—	—	—	—	—	—
DG-(190–204)	99	18,066	558	10,727	10,517*	112,146	81	3554
	223	3,498	301	3,172				
	82	1,781	154	1,410				
	231	1,515	307	1,963				
	254	2,104	425	2,597				
	276	941	4833	25,934				
DG-(206–220)	—	—	106	4,848	10,674	62,438	—	—
			2705	11,210	12,296	57,851		
			2138	7,634	20,590	66,995		
DG-(251–265)	—	—	116	3,943	—	—	—	—
			188	1,641				
DG-(512–526)	—	—	—	—	—	—	—	—
DG-(762–786)	121	5,782	—	—	—	—	—	—
	31	1,128						
	693	2,443						

T-cell lines specific for DG peptides were established from four PV patients with active disease. Blood mononuclear cells were plated at 10^5 cells per well of a U-bottom microtiter plate; typically 24 wells were set up for each individual peptide (5 μM peptide). rIL-2 was added on day 3 and the specificity of T-cell lines was examined in a proliferation assay on days 12–14. T-cell lines with a stimulation >3 were considered positive. T-cell lines with a strong proliferative response to DG-(190–204) (stimulation index >10) were obtained from each patient. No T-cell responses were seen with peptides DG-(78–93), DG-(97–111), and DG-(512–526). Ten wells were set up for each peptide with cells from patient DC, 24 wells per peptide for the other PV cases. T-cell lines specific for DG-(190–204) were set up twice for patient Go (24 wells in the first experiment, 96 in the second) and a total of six DG-(190–204)-specific lines was obtained. The MHC haplotypes of the patients were as follows: patient Go, DRB1*0402, DQB1*0302; patient DC, DRB1*0402, DRB1*0403, DQB1*0302; patient St, DRB1*0402, DRB1*1401, DQB1*0302, DQB1*05031; patient Ro, DRB1*03011, DRB1*1401, DQB1*0201, DQB1*05031.

*The background was high with this patient as autologous Epstein–Barr virus (EBV)-transformed B cells were used as antigen presenting cells for this experiment, while autologous nontransformed mononuclear cells were used in the other cases.

addressed by generating T-cell clones specific for recombinant DG and by determining the epitope specificity of these clones. Since the DRB1*0402 haplotype is uncommon in the general population (≈1% of the general population in the United States), T-cell reactivity to DG and DG peptides can be compared between patients and healthy family members who share the DRB1*0402 haplotype.

Selective Presentation of a DG Peptide by the PV-Linked DR4 Molecule (DRB1*0402). DG-(190–204) and DG-(206–220) specific T-cell lines were HLA-DR restricted as T-cell proliferation was blocked by a monoclonal antibody (mAb) specific for HLA-DR (L243) but not by a mAb specific for HLA-DQ (G2a.5, specific for DQ1, DQ7, and DQ8) (data not shown). T-cell lines specific for DG-(190–204) were cloned by limiting dilution to determine if presentation of this peptide was selective for the PV-linked DRB1*0402 molecule. The DG-(190–204) peptide was presented only by a B-cell line homozygous for DRB1*0402 but not by B cells homozygous for the RA-associated haplotypes (DRB1*0401 and 0404) or other DR4 haplotypes (DRB1*0403, 0405, 0406, 0407, 0408) (Fig. 2). Selective presentation of DG-(190–204) by DRB1*0402 was confirmed using L cell transfectants that expressed DRB1*0401, 0402 or 0404 (Fig. 3). The fact that the peptide was presented by DRB1*0402 but not by DRB1*0404 was particularly informative as these molecules differ only at three polymorphic positions, DRβ 67, 70, and 71.

Residues DRβ 70 and 71 of the P4 Pocket Are Responsible for Selective Presentation of a DG Peptide. Residues critical for peptide presentation were determined by site-directed mutagenesis of DRβ 67, 70, and 71 in the DRB1*0402 cDNA (26). T-cell recognition of the peptide was abolished when glutamic acid at DRβ 71 was replaced by a positively charged amino acid, lysine (as in 0401 or 0409) or arginine (other DR4 subtypes) (Fig. 4). The peptide was also not presented when aspartic acid at DRβ 70 was replaced by glutamine (present in the majority of DR4 subtypes). Substitution of isoleucine at DRβ 67 by leucine only resulted in a moderate reduction of the T-cell response. Thus, two negatively charged residues of the P4 pocket (DRβ 70 and 71) are critical for presentation of DG-(190–204), providing a structural basis for DRB1*0402-linked autoimmunity to PV.

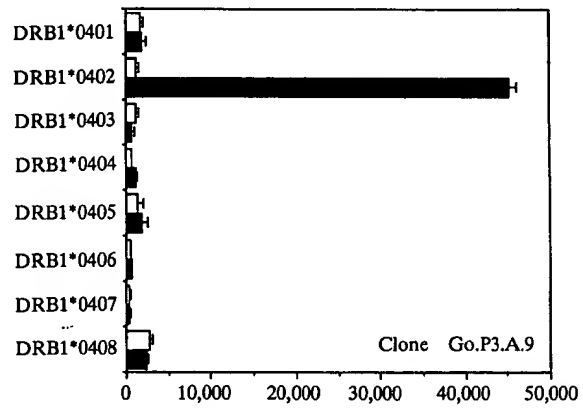

FIG. 2. Presentation of DG-(190–204) is selective for the PV-linked DR4 molecule (DRB1*0402). EBV-transformed B-cell lines [Priess (DRB1*0401), YAR 634 (DRB1*0402), SSTO (DRB1*0403), PE 636 (DRB1*0404), JH22798 (DRB1*0405, DRB1*1301), IUU 020 (DRB1*0406), JHAF (DRB1*0407), and MT 706 (DRB1*0408, DRB1*0404)] were used as antigen-presenting cells for a DG-(190–204)-specific T-cell clone. B cells were pulsed for 18 hr with 20 μM DG-(190–204), washed, irradiated, and cocultured for 3 days with DG-(190–204)-specific T-cell clones (5×10^4 T cells and B cells per well, in triplicates). □, No peptide; ■, plus DG-(190–204). T-cell proliferation was determined by ^{3}[H]thymidine incorporation. Presentation of the DG-(190–204) peptide was specific for the PV-linked DRB1*0402 molecule. DRB1*0402 differs only at three positions from the DRB1*0404 molecule associated with susceptibility to RA, indicating that residues DRβ 67, 70, and 71 are important in defining susceptibility to these two different autoimmune diseases.

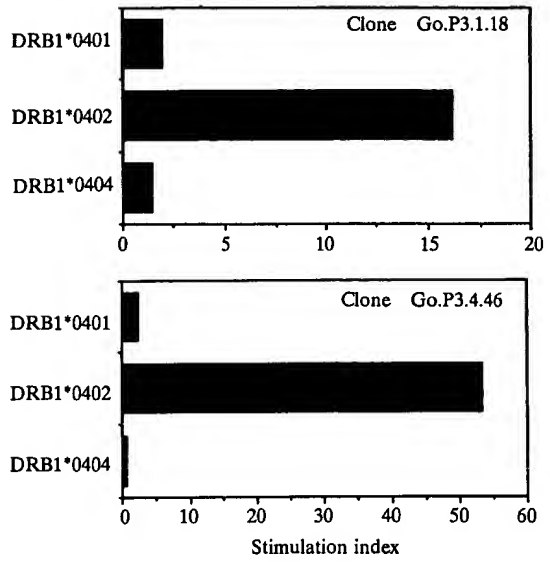

FIG. 3. Presentation of DG-(190–204) by DRA, DRB1*0402 transfectants. L cells expressing the PV-associated DRB1*0402 or the RA-associated DRB1*0401 or 0404 molecules were used as antigen-presenting cells for two DG-(190–204)-specific T-cell clones from a PV patient. L-cell transfectants were pulsed with the DG-(190–204) peptide at 10 μM, washed, irradiated, and cocultured with DG-(190–204)-specific T-cell clones for 72 hr (10^5 L cells, 5×10^4 T cells per well, in triplicates). T-cell proliferation was quantitated by ^{3}[H]thymidine incorporation. Numbers represent the stimulation index (cpm in the presence of peptide/cpm in the absence of peptide).

T-Cell Clones Specific for DG (Residues 190–204) Secrete IL-4 and IL-10. DG-specific autoantibodies interfere with keratinocyte cell adhesion and thereby induce the severe skin blistering seen in PV patients (12). Induction of autoantibody production by DG-specific T cells would require the production of cytokines that induce the activation and differentiation of autoreactive B cells. IL-4 and IL-10 are important cytokines that are secreted by Th2 cells and that promote B-cell differentiation and antibody production (reviewed in ref. 27).

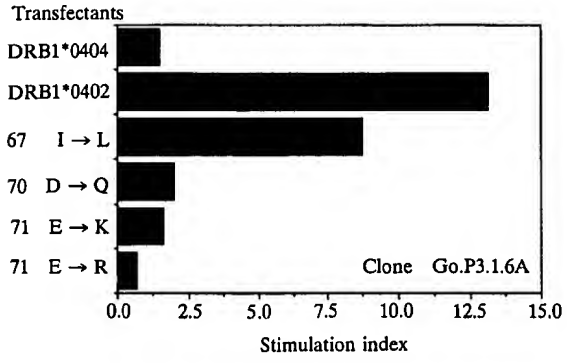

FIG. 4. Residues 70 and 71 of the DRβ chain are responsible for the selective presentation of DG-(190–204) by DRB1*0402. The DG-(190–204) peptide was presented by the PV-associated DRB1*0402 molecule but not by the RA-associated DRB1*0404 molecule, which differ only at DRβ 67, 70, and 71. DRβ 67, 70, and 71 of DRB1*0402 were mutated to those residues found in DRB1*0404. L-cell transfectants that expressed these molecules were used as antigen-presenting cells in a T-cell proliferation assay. Numbers represent the stimulation index (cpm in the presence of peptide/cpm in the absence of peptide). The data demonstrate that DRβ 70 and 71 of the P4 pocket confer selective presentation of the DG-(190–204) peptide.

To examine antigen-driven cytokine production of the DG-(190–204)-specific T-cell clones, T cells were cocultured with autologous blood mononuclear cells as antigen-presenting cells. Cytokine content of supernatants was monitored after 48 hr by ELISA. Two of three clones tested secreted high levels of IL-4; one of the clones also secreted large amounts of IL-10 (Table 4). No detectable quantities of IL-4 or IL-10 were secreted in the absence of antigen. Antigen-driven IL-4 and IL-10 secretion by these T-cell clones may be important in the activation and differentiation of B cells that produce DG-specific autoantibodies.

DISCUSSION

Polymorphic residues that shape the P4 pocket of HLA-DR4 molecules appear to be critical in determining susceptibility to two different autoimmune diseases, RA and PV. Why is the P4 pocket so important? The P4 pocket is located in a central position of the HLA-DR peptide binding site and is flanked by residues that act as primary T-cell receptor contact residues (P2, P3, and P5, as demonstrated for an immunodominant myelin basic protein peptide) (7, 23, 28, 29). The P4 pocket has a surface that appears to be extremely variable (only 23% conserved) while the surface of the P6 pocket is more conserved (49%) (33). In contrast, the P1 pocket is shaped mostly by residues of the nonpolymorphic DRα chain and is occupied by hydrophobic anchor residues in all DR molecules (6, 7). Among the polymorphic residues of the P4 pocket, the charge at DRβ 71 is critical for the selective binding of self-peptides to the RA- and PV-linked DR4 molecules (4, 10). The negative charge of DRβ 70 and 71 in the PV-linked DRB1*0402 molecule confers selective binding to self-peptides that have a positive charge at P4 [a lysine in DG-(190–204) and an arginine in DG-(206–220)]. The positive charge of DRβ 71 in the RA-associated DR4 molecules confers selective binding of peptides with a negative charge at P4.

The self-peptides recognized by autoaggressive T cells in human autoimmune diseases have been difficult to identify. For many of the common autoimmune diseases (RA, insulin-dependent diabetes, multiple sclerosis) the target antigens are not known, although candidate antigens have been identified (i.e., type II collagen in RA, glutamic acid decarboxylase 65 in type I diabetes, and myelin basic protein and proteolipid protein in multiple sclerosis). The identity of target proteins has been established for those autoimmune diseases in which autoantibodies interfere with specific cellular functions (i.e., neuromuscular transmission in myasthenia gravis, control of thyroid hormone secretion in Grave disease) (30). T-cell

Table 4. Antigen-driven IL-4 and IL-10 secretion was examined for three DG-(190–204)-specific T-cell clones from a PV patient

Clone	No antigen	20 μM	10 μM	5 μM
	IL-4 secretion, pg/ml			
Go.P3.1.6A	<10	980	330	145
Go.P3.4.22	<10	190	103	<10
Go.P3.A.9	<10	420	330	560
	IL-10 secretion, pg/ml			
Go.P3.1.6A	<10	1450	630	450
Go.P3.4.22	<10	180	95	77
Go.P3.A.9	<10	78	148	230

T cells (5×10^4 per well) were cocultured with autologous mononuclear cells (10^5 per well) for 48 hr; synthetic peptide was added to 5, 10, or 20 μM. Cytokine content of supernatants was determined by sandwich ELISA (PharMingen) using rIL-4 and rIL-10 as standards. Two T-cell clones secreted large amounts of IL-4; one of the clones also secreted large quantities of IL-10. Cytokine secretion was not detectable in the absence of antigen. Antigen-driven IL-4 and IL-10 secretion by DG-specific T cells may be important in inducing the production of DG-specific autoantibodies in PV.

recognition of some of these autoantigens has been studied; however, it is not clear which of the T-cell epitopes identified are important in the disease process as no clear structural relationship between the disease-associated MHC molecules and self-peptides from these autoantigens has emerged. In PV, the situation is unique because (*i*) the autoantigen has been identified and the immunological effector mechanisms have been established (11–15), (*ii*) the linkage of disease susceptibility to the DRB1*0402 or the DQB1*0503 haplotype is very strong (9, 17–19), and (*iii*) unique structural features of the DRB1*0402 peptide binding site can be defined since there are many structurally related DR4 subtypes that are not associated with the disease (Table 1). The fact that the disease-linked polymorphisms shape the specificity of a pocket of the DRB1*0402 peptide binding site strongly suggests that peptide presentation to T cells is important in the pathogenesis of PV. The observation that a DG peptide with a positive charge at P4 is presented by DRB1*0402 but not by other DR4 subtypes may explain why the disease is associated with this particular MHC class II molecule.

A striking aspect of the two DR4-linked autoimmune diseases is that RA and PV are clinically and immunologically so different, even though the disease-associated DR4 molecules differ in a small, albeit strategic, location. In RA the joint destruction is thought to result from a chronic Th1-mediated autoimmune reaction against the synovial lining of the joint (31); in PV the disease is probably caused by Th2 cells that induce the production of DG-specific autoantibodies (11). In the skin, keratinocytes can act as antigen-presenting cells since they express MHC class II molecules following exposure to γ-interferon. Keratinocytes that express MHC class II molecules (i.e., following local inflammation or UV exposure) may therefore present DG peptides to T cells; Langerhans cells of the skin may also be involved in the presentation of the autoantigen. Keratinocytes are known to skew T-cell responses to a Th2 cytokine profile (IL-4 and IL-10) due to a failure to produce sufficient quantities of IL-12 (reviewed in ref. 32). The local mechanisms of antigen presentation in the skin and in the joint may therefore be critical in inducing Th2- or Th1-mediated autoimmune responses that produce such different clinical manifestations in PV and RA.

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